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Age-dependent impairment of metabotropic glutamate receptor 2-dependent long-term depression in the mouse striatum by chronic ethanol exposure

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Abstract

Chronic alcohol exposure is associated with increased reliance on behavioral strategies involving the dorsolateral striatum (DLS), including habitual or stimulus-response behaviors. Presynaptic G protein-coupled receptors (GPCRs) on cortical and thalamic inputs to the DLS inhibit glutamate release, and alcohol-induced disruption of presynaptic GPCR function represents a mechanism by which alcohol could disinhibit DLS neurons and thus bias towards use of DLS-dependent behaviors. Metabotropic glutamate receptor 2 (mGlu₂) is a G_{i/o}-coupled GPCR that robustly modulates glutamate transmission in the DLS, inducing long-term depression (LTD) at both cortical and thalamic synapses. Loss of mGlu₂ function has recently been associated with increased ethanol seeking and consumption, but the ability of alcohol to produce adaptations in mGlu₂ function in the DLS has not been investigated. We exposed male C57Bl/6J mice to a two-week chronic intermittent ethanol (CIE) paradigm followed by a brief withdrawal period, then used whole-cell patch clamp recordings of glutamatergic transmission in the striatum to assess CIE effects on mGlu₂-mediated synaptic plasticity. We report that CIE differentially disrupts mGlu₂-mediated long-term depression in the DLS vs. dorsomedial striatum (DMS). Interestingly, CIE-induced impairment of mGlu₂-LTD in the dorsolateral striatum is only observed when alcohol exposure occurs during adolescence. Incubation of striatal slices from CIE-exposed adolescent mice with a positive allosteric modulator of mGlu₂ fully rescues mGlu₂-LTD. In contrast to the two-week CIE paradigm, acute exposure of striatal slices to ethanol concentrations that mimic ethanol levels during CIE fails to disrupt mGlu₂-LTD. We did not observe a reduction of mGlu₂ mRNA or protein levels following CIE, suggesting that alcohol effects on mGlu₂ occur at the

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functional level. Our findings contribute to growing evidence that adolescents are uniquely vulnerable to certain alcohol-induced neuroadaptations, and identify enhancement of mGlu₂ activity as a strategy to reverse the effects of adolescent alcohol exposure on DLS physiology.

Keywords

chronic intermittent ethanol exposure; basal ganglia; synaptic transmission; synaptic plasticity; adolescent alcohol

Introduction

Chronic alcohol exposure produces maladaptive behaviors that reflect increased engagement of dorsolateral striatum (DLS)-dependent action strategies such as habitual alcohol seeking (Barker et al., 2015; Corbit and Janak, 2016; Gremel and Lovinger, 2016). Loss of flexible, goal-directed control over alcohol seeking is likely to contribute to high levels of alcohol consumption after long periods of alcohol exposure. Animals exposed to alcohol prior to training in instrumental learning tasks for either alcohol or natural reinforcers show increased reliance on habitual response strategies (Corbit et al., 2012, 2014; Dickinson et al., 2002; Renteria et al., 2018), which are known to depend on DLS activity (Balleine et al., 2009; Yin et al., 2004). Habitual alcohol seeking in rats is reduced by pharmacological inactivation of the DLS or blockade of fast glutamate transmission within the DLS (Corbit et al., 2012, 2014), suggesting that enhanced glutamatergic drive to this region, which primarily arises from sensorimotor cortical regions and the intralaminar nuclei of the thalamus (Hunnicutt et al., 2016), could account for the predominance of DLS-dependent response strategies following alcohol exposure. In addition to enhanced habit formation, prior alcohol exposure also promotes other DLS-dependent learning such as improved performance in a visual discrimination task (DePoy et al., 2013), and this is associated with increased firing of DLS neurons during learning. Identification of adaptations in DLS physiology that promote the use of DLS-dependent behavioral strategies will provide critical opportunities to design interventions aimed at reducing habitual alcohol seeking.

Glutamatergic inputs to medium spiny neurons (MSNs), the major projection neurons of the striatum, are critical determinants of MSN activity. Presynaptic G_{i/o}-activating G protein-coupled receptors (GPCRs) modulate the strength of glutamatergic inputs to the striatum by reducing glutamate release. Impaired presynaptic regulation of glutamate release in afferents to the DLS represents a mechanism by which DLS activity could be enhanced to promote the use of habitual action strategies. Adaptations in GPCR function caused by alcohol and other drugs of abuse have been well-documented, including impaired CB1 cannabinoid receptor and mu opioid receptor function in glutamatergic inputs to the striatum following alcohol exposure (Adermark et al., 2011; DePoy et al., 2013; DePoy et al., 2015; Johnson and Lovinger, 2016; Munoz et al., 2018; Xia et al., 2006). Metabotropic glutamate receptor 2 (mGlu₂) is a particularly robust modulator of glutamatergic transmission in the striatum, and several recent studies have implicated reduced mGlu₂ expression as a driver of increased alcohol intake and seeking (Meinhardt et al., 2013; Zhou et al., 2013). However, these studies did not specifically assess the physiological roles of mGlu₂ in the dorsal striatum and

how those are altered by EtOH. We and others have shown previously that in the DLS, brief treatment of striatal slices with a group II mGlu receptor agonist results in an mGlu₂-dependent, presynaptically-mediated form of long-term depression (LTD) of electrically-evoked glutamate transmission (Johnson et al., 2017; Kahn et al., 2001; Kupferschmidt and Lovinger, 2015). In contrast to LTD induced by activation of CB1 or mu opioid receptors, mGlu₂-LTD occurs at both cortical and thalamic inputs to the striatum (Atwood et al., 2014; Johnson et al., 2017; Wu et al., 2015), suggesting that mGlu₂ is poised to exert broad inhibitory control of striatal activation. Loss of presynaptic inhibition of glutamate transmission onto MSNs in the DLS could contribute to alcohol-induced bias towards use of DLS-dependent behavioral strategies. Currently, it is unknown if alcohol exposure disrupts mGlu₂ function in glutamatergic neurons that drive DLS activity.

In this study, we aimed to determine if mGlu₂-mediated presynaptic depression of glutamate transmission in the DLS is impaired by alcohol exposure. Because there are differences in vulnerability to alcohol across the lifespan (Spear and Swartzwelder 2014, Crews 2016), we assessed alcohol effects on mGlu₂-LTD in both adolescent and adult mice. We report that adolescent mice subjected to a chronic intermittent ethanol vapor exposure (CIE) paradigm are uniquely vulnerable to alcohol-induced disruptions of mGlu₂-mediated synaptic plasticity in the dorsal striatum. Using whole-cell patch clamp recordings in acute striatal slices, we find that CIE impairs the long-term, but not the early component of mGlu₂-mediated depression of glutamate transmission in the DLS. Incubation of striatal slices from alcohol-naïve adolescent mice with ethanol does not mimic the effect of CIE on mGlu₂-LTD. In contrast to our findings in the DLS, CIE only modestly reduces the magnitude of mGlu₂-LTD in the dorsomedial striatum (DMS). Finally, we demonstrate that in slices from CIE mice, mGlu₂-LTD in the DLS can be rescued by acute application of a positive allosteric modulator of mGlu₂, which has important implications for future efforts to correct alcohol-induced adaptations in synaptic plasticity using pharmacological interventions.

Materials and Methods

Animals

Animals were housed in the Fishers Lane Animal Care facility managed by the National Institute on Alcoholism and Alcohol Abuse (NIAAA). Studies were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals and were approved by the NIAAA Animal Care and Use Committee. Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME; Stock No. 000664) were used for all alcohol exposure experiments. All subjects were allowed to acclimate to the facility for at least one week prior to onset of alcohol exposure. Mice with a global deletion of the gene encoding mGlu₂ (Grm2) were used for mGlu₂ antibody validation. Mice were housed on 12 hour light/dark cycle on ventilated racks in a temperature- and humidity-controlled room with *ad libitum* access to food and water.

Generation of Grm2 knockout mice

CRISPR/Cas9 based mutagenesis was used to create Grm2 gene knockout (Fig. S1a) and floxed mice. Floxed mouse production will be described elsewhere. [Benchling.com](https://www.benchling.com) was

used to identify sgRNA binding sites in introns 2 and 3 of the *Grm2* locus (Fig. S1b). Cas9 mRNA (Cong et al., 2013) and tru-sgRNAs (Fu et al., 2014) were produced by *in vitro* transcription as described (Bassett et al., 2013; Blednov et al., 2017). sgRNAs (75 ng/μl), Cas9 mRNA (50 ng/μl), and loxP targeting oligonucleotides (50 ng/μl) were microinjected into the cytoplasm of C57BL/6J one-cell mouse embryos using standard procedures (Blednov et al., 2017; Yang et al., 2014). One of the founder mice obtained was mosaic and transmitted the floxed allele or a deletion allele to offspring. No off-target mutations were detected in the founder for the top 9 sites that were predicted by Benchling for either sgRNA. The deletion allele removed 1843 bp from the *Grm2* locus including Exon 3 (838 bp) and inserted 27 bp that includes a partial loxP sequence (Fig. S1c). Deletion of Exon 3 is predicted to create a frameshift mutation. Mice were maintained on an inbred C57BL/6J genetic background. Mice were genotyped with PCR. WT specific F1 (5'-CAGATTCTGCTGGCCCATGA-3') and R1 (5'-TTGGTCTCCA TTGGA TGCCC-3') primers produced a 420 bp amplicon. Primer F1 was used with R2 (5'-CACCTAAATAGAAGTTCTCCC-3') to produce a 336 bp amplicon that was specific to the KO allele (note: the 2152 bp amplicon from the WT allele with this primer set was not consistently observed).

Chronic intermittent ethanol vapor exposure (CIE) and withdrawal

Mice were exposed to two rounds of ethanol vapor or air (control condition). Rounds consisted of four consecutive 16 hour vapor or air exposure sessions (5 PM – 9 AM, Monday evening through Friday morning) separated by 8 hours of withdrawal. For late adolescent alcohol exposure, the first cycle of CIE began when mice were 6 weeks old. Adult CIE began when mice were 12–14 weeks old. During exposure, mice remained in their homecage with the lid removed to allow air flow into the cage. Wire racks containing food and water bottles remained in place. Cages were placed in Plexiglas chambers (Plas Labs Inc., Lansing, MI). Air was bubbled through 95% ethanol at a rate of 2–3 L/min to volatilize the ethanol. Ethanol vapor was then combined with another air stream for a total flow rate of ~10 L/min into the chamber. Controls were exposed to room air at the same flow rate. Ethanol levels were adjusted so that an air sample from the chamber resulted in ethanol concentrations of 0.175–0.200 mg/L. Mice were not given pyrazole or ethanol loading injections prior to placement in the vapor chambers. Throughout the two rounds of alcohol exposure, mice were housed in the room containing the vapor chamber system to avoid unnecessary stress associated with transport. The same light cycle, temperature, and humidity controls as the primary housing room were maintained in this satellite room. Mice were restrained and tail blood was collected at the end of the 3rd or 4th ethanol exposure, and blood ethanol concentrations were determined according to manufacturer instructions using an Analox Alcohol Analyzer (Analox Instruments, UK) or an alcohol detection assay kit (Pointe Scientific, Canton, MI). Mice were sacrificed for electrophysiology experiments or tissue harvesting for gene expression analysis 3–6 days after the last ethanol exposure session unless otherwise noted.

Brain slice preparation

Acute brain slices (coronal, 250 μm thick) were prepared using a vibratome V1200S (Leica Microsystems) as previously described (Johnson et al., 2017). Mice were anesthetized with

isoflurane prior to decapitation, and brains were rapidly removed and immediately submerged in ice-cold cutting solution containing (in mM): 30 NaCl, 4.5 KCl, 1 MgCl₂, 26 NaHCO₃, 1.2 NaH₂PO₄, 10 D-glucose, and 194 sucrose, continuously bubbled with 95% O₂/5% CO₂. Slices were placed in a 32°C holding chamber filled with artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 26 NaHCO₃, 1.2 NaH₂PO₄, and 10 D-glucose, 305–310 mOsm, continuously bubbled with 95% O₂/5% CO₂. Slices were allowed to recover for 30 minutes at 32°C, and then were incubated at room temperature for 1–6 hours prior to beginning experiments.

Whole-cell voltage clamp electrophysiology recordings and analysis

Whole-cell voltage-clamp recordings were performed as previously described (Johnson et al., 2017). Hemisected slices were submerged in a diamond-shaped recording chamber (Warner Instruments) and were continuously perfused with 30–32°C aCSF at a rate of ~1.5 mL/min. Recording pipettes (2.0–4.0 MΩ resistance in bath) were filled with Cs-based internal solution (295–300 mOsm) containing (in mM): 120 CsMeSO₃, 5 NaCl, 10 TEA-Cl, 10 HEPES, 5 QX-314, 1.1 EGTA, 0.3 Na-GTP, and 4 Mg-ATP; pH was adjusted to 7.2–7.3 using CsOH. Visualization was performed on a Zeiss Axioskop 2 microscope using a 40x/0.8 NA water-immersion objective. Putative MSNs were identified based on size, capacitance, and membrane resistance. Recordings were performed using a Multiclamp 700A amplifier (Axon Instruments). Cells were voltage-clamped at –60 mV, and a parallel bipolar stimulating electrode (~100 μm tip separation) was used to produce electrically-evoked excitatory postsynaptic currents (EPSCs) at 0.05 Hz. For both DLS and DMS recordings, stimulating electrode tips were placed in the striatum just inside the external capsule and ~100 μm from the recorded cell. Stimulation intensity was typically adjusted to elicit 200–600 pA current amplitudes. Acquisition was performed using Clampex 10.3 (Molecular Devices). Access resistance was monitored during recordings and only cells with a stable access resistance (less than 20% change from baseline) were included for analysis. Recordings were filtered at 2 kHz and digitized at 10 kHz (Digidata 1322A, Axon Instruments). For all recordings, 50 μM picrotoxin was included in the aCSF to block fast GABAergic transmission.

EPSC amplitudes were measured using Clampfit 10 software (Molecular Devices). GraphPad Prism 7.0 was used to prepare graphs and perform statistical analyses. EPSC amplitudes were averaged each minute and normalized to the average EPSC amplitudes during a stable 10 minute baseline recording. Early drug effects were measured at 8–10 minutes after the onset of LY379268 application, and LTD magnitude was measured at 26–30 minutes after onset of LY379268 application. Sample traces and % baseline values reported in the text are averaged from these time windows. In some cases, stimulation artifacts in sample traces were truncated for clarity. All data are reported as mean ± SEM. *N* values represent the number of recorded cells. One experiment was performed on each slice. Each reported condition contains experiments obtained from at least 2 animals, but typically more, and from at least two cohorts of CIE-exposed mice when applicable. Comparisons of early depression or LTD magnitude between groups were made using an unpaired *t* test or a two-way ANOVA followed by post-hoc Tukey's test for multiple comparisons. Alpha was set at 0.05.

Drugs and treatments

Picrotoxin, QX-314, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). LY379268 was purchased from Tocris (Minneapolis, MN) and stock solutions were prepared at 10 mM in 0.01 N NaOH. mGlu₂-LTD was induced by a 5 min application of 100 nM of LY379268 (diluted in aCSF). LY487379 was purchased from Tocris and stock solutions were prepared at 10 mM in DMSO. LY487379 or control DMSO concentration (0.1%) were diluted in aCSF and held constant throughout the experiment. In our experience, this concentration of DMSO does not affect the magnitude or time course of mGlu₂-LTD. 95% ethanol was purchased from the U.S. Department of Health and Human Services Supply Service Center.

Quantitative polymerase chain reaction (qPCR) and analysis

Mice were anesthetized with isoflurane and decapitated, and brains were removed and rinsed in cold 0.9% NaCl. One mm coronal sections were prepared using a stainless steel brain matrix, and samples from primary motor cortex, agranular insular cortex, medial prefrontal cortex, and thalamus were placed in RNAlater (Invitrogen), stored at 4°C for 48 hours, then removed from RNAlater and frozen at -20°C prior to RNA extraction. RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen), and RNA concentration was measured using a NanoDrop (Thermo Fisher Scientific). RNA (100–140 ng) was reverse transcribed using the Quantitect Reverse Transcription Kit (Qiagen). qPCR reactions contained 2 µl cDNA template, 1 µl predesigned TaqMan Gene Expression Assay (Mm01235832), 10 µl TaqMan Fast Advanced Master Mix (both from Thermo Fisher Scientific), and 7 µl Ultrapure water (Invitrogen). qPCR was run on a StepOnePlus system (Thermo Fisher Scientific) and was performed according to the manufacturer's recommended settings: 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles of melting at 95 °C for 1 sec and annealing/extension at 60 °C for 20 sec. Reactions were run in triplicate for 7–8 animals per group. Analysis was performed using the 2^{-CT} method (Schmittgen and Livak, 2008). The C_T value was determined by identifying the cycle number at which the amplification curve reach a R_n threshold set at 0.1 (R_n = R_n – baseline, where R_n is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye). Relative mGlu₂ expression was quantified using Actb as an internal control (Applied Biosystems #4351315). Thus, C_T = C_T (mGlu₂) – C_T (Actb). Between-group comparisons were performed using an unpaired t test.

Protein expression analysis

Mice were anesthetized with isoflurane and decapitated, and brains were removed and rinsed in cold 0.9% NaCl. One mm coronal sections were prepared using a stainless steel brain matrix, and bilateral 1 mm punches from DLS and DMS were placed in microcentrifuge tubes and immediately frozen on dry ice, then stored at -80°C until protein extraction. Samples were homogenized on ice in 50 µL of RIPA (ThermoFisher Scientific) buffer containing 1x Halt TM Protease Inhibitor Cocktail (ThermoFisher Scientific) and centrifuged at 20,000 x g for 20 min at 4°C. Protein concentration was determined using the Pierce TM BCA protein assay (ThermoFisher Scientific) according to the microplate procedure described in the manufacturer's instructions. Protein concentrations were

calculated from a standard curve fit to a quadratic function. mGlu₂ protein expression was quantified using the Wes TM capillary electrophoresis system (ProteinSimple®) according to the manufacturer's instructions with the exception that samples were denatured at room temperature to minimize protein aggregation common to GPCRs. Preliminary experiments were conducted to determine optimal primary antibody and protein concentrations. Assays were run using 0.5 µg/µL total protein, monoclonal rabbit anti-mGlu₂ antibodies (1:500, D7D8M mAB#76012, Cell Signaling Technology) and HRP-conjugated goat anti-rabbit secondary antibodies (1x, ProteinSimple®). The specificity of the anti-mGlu₂ antibodies was determined using protein lysates from the striatum of mGlu₂ global knockout mice. mGlu₂ immunoreactivity was observed as two peaks in the electrophoretogram positioned at approximately 112 kDa and 180 kDa. Each sample was analyzed in duplicate and mGlu₂ immunoreactivity was quantified by integrating the chemiluminescence signal from 78 kDa to 345 kDa and normalizing to total protein.

Results

CIE does not disrupt mGlu₂-LTD in the DLS of adult mice

To investigate the effects of alcohol exposure on mGlu₂-mediated depression of glutamatergic transmission in the DLS, we used a CIE protocol in which mice were placed in ethanol vapor exposure chambers or control air exposure chambers for 16 hours per day, four days per week, for two cycles, with 3 days of withdrawal between cycles. Mice were allowed to withdraw from ethanol for 3–6 days prior to preparation of brain slices for *ex vivo* electrophysiology recordings. Blood ethanol concentrations measured at the end of the third or fourth 16 hour exposure session were 185.1 ± 8.3 mg/dL ($n = 7$). We performed whole-cell voltage clamp recordings of electrically-evoked excitatory postsynaptic currents (EPSCs) from MSNs in the DLS to determine the effect of our CIE paradigm on mGlu₂-LTD. In adult mice, the CIE protocol did not alter depression of EPSC amplitudes induced by bath application of the mGlu_{2/3} agonist LY379268 (100 nM, 5 min) at early or late time points following drug application (early depression $60.6 \pm 7.0\%$ of baseline in controls vs. $42.7 \pm 7.2\%$ of baseline in CIE mice, $p = 0.11$, unpaired *t* test; late depression $38.6 \pm 3.9\%$ of baseline in controls vs. $48.9 \pm 9.5\%$ of baseline in CIE mice, $p = 0.36$) (Fig. 1).

CIE impairs mGlu₂-LTD in the DLS of adolescent mice

Age-dependent vulnerability to alcohol-induced neuroadaptations has been identified for a number of cellular, physiological, and behavioral measures (Crews et al., 2016; Spear and Swartzwelder, 2014). Thus, we measured mGlu₂-mediated depression of electrically-evoked glutamate transmission in the DLS following CIE administered during late adolescence (two cycles of CIE beginning at 6 weeks of age). Blood ethanol concentrations measured at the end of the third or fourth 16 hour exposure session were 184.1 ± 12.7 mg/dL ($n = 13$), which was not significantly different from ethanol concentrations measured from adult mice ($p = 0.96$, unpaired *t* test). CIE caused a disruption of the long-term, but not early component of LY379268-induced depression of transmission (Fig. 2). Loss of the robust mGlu₂-LTD observed in alcohol-naïve mice could be caused by reduced mGlu₂ signaling due to lower expression levels or impaired coupling to heterotrimeric G proteins, both of which could be rescued by enhancing receptor activation with a positive allosteric modulator (Nickols and

Conn, 2014). Thus, we evaluated the effects of the mGlu₂-selective positive allosteric modulator LY487379 (10 μ M, present throughout recording) on mGlu₂-LTD in slices from both air and CIE mice. Acute application of LY487379 restored mGlu₂-LTD in CIE-treated mice (Fig. 2). Two-way ANOVA did not reveal a main effect of either CIE or PAM treatment on the early component of LY379268-induced depression of EPSCs (CIE: $F_{(1,23)} = 0.48$, $p = 0.50$; PAM: $F_{(1,23)} = 0.002$, $p = 0.96$; interaction $F_{(1,23)} = 0.38$, $p = 0.54$) (Fig. 2a–e). For the late component of LY379268-induced depression, two-way ANOVA revealed a main effect of CIE ($F_{(1,23)} = 8.46$, $p = 0.008$), a main effect of PAM ($F_{(1,23)} = 12.14$, $p = 0.002$), and a significant CIE $\times$ PAM interaction ($F_{(1,23)} = 6.12$, $p = 0.02$) (Fig. 2a–d, f). *Post hoc* comparisons revealed that in slices without PAM treatment, CIE caused an impairment of LY379268-induced LTD, resulting in a substantial reversal of EPSC inhibition following the initial depression (air $46.3 \pm 7.3\%$ of baseline; CIE $83.2 \pm 5.9\%$ of baseline; $p = 0.0009$, Tukey's multiple comparisons test) (Fig. 2a, d). Treatment of slices from air controls with the mGlu₂-selective PAM LY487379 (10 μ M, present throughout recording) did not enhance the magnitude of LY379268-induced LTD ($39.4 \pm 3.5\%$ of baseline with PAM vs. $46.3 \pm 7.3\%$ of baseline without PAM, $p = 0.91$) (Fig. 2c–e). However, LY487379 rescued mGlu₂-LTD in CIE slices ($42.4 \pm 5.8\%$ of baseline with PAM vs. $83.2 \pm 5.9\%$ of baseline without PAM, $p = 0.0007$) (Fig. 2c, d, f). The magnitude of LTD measured in slices from CIE mice in the presence of PAM did not substantially differ from that observed in control mice in the absence of PAM ($p = 0.97$) (Fig. 2f).

In our initial experiments, CIE administered during adolescence impaired mGlu₂-LTD induction measured 3–6 days after the last ethanol exposure session, but it was unclear if this withdrawal period was required for alcohol to disrupt LTD. To test this, we evaluated the effects of CIE on mGlu₂-LTD in slices prepared from mice immediately after completing the final exposure session. Similar to the results obtained following more prolonged withdrawal, CIE did not affect the early depression of EPSCs by LY379268 ($40.9 \pm 5.8\%$ of baseline for controls vs. $53.2 \pm 7.4\%$ of baseline for CIE, $p = 0.22$, unpaired *t* test) (Fig. 3a–c), whereas the magnitude of mGlu₂-LTD was significantly impaired by CIE ($42.9 \pm 3.7\%$ of baseline for controls vs. $77.8 \pm 9.7\%$ of baseline for CIE, $p = 0.0072$) (Fig. 3a, b, d).

Acute ethanol does not disrupt mGlu₂-LTD

Acute alcohol exposure is also known to alter synaptic physiology, so we asked whether exposing slices obtained from alcohol-naïve mice to alcohol would cause an immediate disruption mGlu₂-LTD in the DLS. We perfused striatal slices with aCSF containing 40 mM ethanol to mimic the blood ethanol concentrations measured in our CIE paradigm (mean 41.2 mM). When ethanol was present throughout the recording, LY379268 still produced robust LTD of EPSCs in the DLS that was similar in magnitude to LTD observed in control animals in the absence of ethanol ($40.4 \pm 5.3\%$ of baseline, $n = 7$) (Fig. 4).

CIE impairs mGlu₂-LTD in the DMS of adolescent mice

Chronic alcohol is known to produce a shift towards reliance on DLS-dependent control of behavior including enhanced habit formation and reversal learning in a visual discrimination task. Impaired mGlu₂-dependent presynaptic inhibition of glutamate release represents a plausible mechanism by which the DLS becomes disinhibited to influence behavior.

However, mGlu₂ activation could also induce LTD of glutamate transmission in the DMS, an area of the striatum that is critical for goal-directed behavioral strategies (Balleine et al., 2009; Yin et al., 2005). We compared the effects LY379268 on electrically-evoked EPSCs recorded from the DMS of adolescent mice exposed to air or CIE and found that similar to effects observed in the DLS, LY379268 application induces LTD of glutamate transmission in the DMS (Fig. 5a). Differences between air and CIE mice during the early component of LY379268-induced synaptic depression did not reach statistical significance ($41.4 \pm 7.4\%$ of baseline for controls vs. $64.2 \pm 7.6\%$ of baseline for CIE, $p = 0.058$, unpaired t test) (Fig. 5a–c). However, CIE did reduce the magnitude of mGlu₂-LTD ($37.3 \pm 6.0\%$ of baseline for controls vs. $59.4 \pm 6.8\%$ for CIE, $p = 0.037$) (Fig. 5a, b, d). Qualitatively, alcohol exposure differentially impaired mGlu₂-LTD in the lateral vs. medial subregions of the striatum. Whereas mGlu₂-mediated depression of EPSCs was almost fully reversible in the DLS of alcohol-exposed mice (Fig. 2a), mGlu₂ activation in the DMS of alcohol-exposed mice produced a smaller but stable form of LTD (Fig. 5a).

CIE effects on mGlu₂ expression in adolescent mice

Several scenarios resulting in decreased receptor function could contribute to alcohol-mediated impairment of mGlu₂-LTD in the striatum. Notably, reduced receptor expression would be predicted to decrease receptor efficacy and could explain the impaired expression of mGlu₂-LTD following alcohol exposure. Because the electrical stimulation used in our electrophysiology recordings evokes glutamate release from a heterogeneous population of inputs arising from various cortical regions and the thalamus, we selected two prominent cortical input regions to the DLS (primary motor cortex and anterior insular cortex), one major DMS input region (medial prefrontal cortex), and the midline thalamus for evaluation of Grm2 levels following CIE. Using reverse transcription followed by quantitative PCR, we did not find evidence that CIE reduced Grm2 expression in any evaluated region (Fig. 6). However, this approach had several limitations that could prevent observation of a relevant decrease in gene expression. First, we did not perform an exhaustive assessment of Grm2 expression across the numerous cortical inputs that could be affected. Second, it is possible that alcohol decreases Grm2 expression in a subpopulation of cortical or thalamic neurons that project to the striatum; therefore, we would not have the required anatomical resolution in our tissue punches to observe more restricted effects on expression. Third, it is possible that downregulation of mGlu₂ could occur at the protein rather than mRNA level. Thus, we also evaluated mGlu₂ protein expression in the DLS and DMS of adolescent air- and CIE-exposed mice. First, we evaluated the specificity of a monoclonal antibody (Cell Signaling Technology) raised against the extracellular loops of mGlu₂. In both DLS and DMS of air and CIE-exposed mice this antibody detected two prominent peaks at 118 and 180 kDa, consistent with the known molecular weights of mGlu₂, with little to no immunoreactivity at lower molecular weights (Figure 7). Despite confirmation that mGlu₂ mRNA was undetectable in mGlu₂ knockout mice (Fig. 7a), we found that ~17% of the signal measured in striatal samples from wild type mice was still present in mGlu₂ knockout mice (Fig. 7b), indicating modest off-target binding of this antibody. Because no other commercially available antibody we tested could reliably and specifically detect mGlu₂ (data not shown), we proceeded to measure mGlu₂ expression in the DLS and DMS of air and CIE mice using this antibody. We did not find evidence of altered levels of mGlu₂ protein in either the DLS

or DMS of CIE mice compared with air controls (Fig. 7c–f). Based on these results, it is unlikely that alcohol-induced changes in mGlu₂ expression at either the transcription or protein level are a major contributor to alcohol-induced disruption of mGlu₂ function in the dorsal striatum.

Discussion

In the present study, we determined that mGlu₂-LTD in the DLS is disrupted after a short withdrawal period only when CIE is administered during adolescence. Mice were still in the late adolescent stage when we assessed alcohol effects on mGlu₂-LTD; thus, to fully understand the consequences of adolescent alcohol-induced deficits in striatal plasticity, future studies will be needed to determine if these effects persist into adulthood following adolescent exposure or if plasticity is only impaired transiently with this treatment regimen. Adolescent alcohol exposure has been shown to cause persistent effects on both physiology and behavior measured in adulthood across a number of brain regions and behavioral domains (Crews et al., 2016; Spear and Swartzwelder, 2014). For example, alcohol administration during adolescence decreases tonic GABA currents, increases NMDA receptor-mediate currents, and decreases stimulation thresholds for induction of long-term potentiation in the hippocampus (Fleming et al., 2012; Risher et al., 2015; Swartzwelder et al., 2017). In the adult prefrontal cortex, dopamine and GABA transmission are disrupted following adolescent alcohol exposure (Centanni et al., 2017; Trantham-Davidson et al., 2017). Imaging studies have demonstrated that adolescent alcohol exposure causes persistent alterations in resting-state functional connectivity, particularly in frontostriatal circuits relevant to selection of action strategies (Broadwater et al., 2018). Behaviorally, adolescent alcohol exposure is associated with persistent increases in voluntary alcohol consumption and enhanced rewarding and reinforcing effects of alcohol during adulthood (Alaux-Cantin et al., 2013; Broadwater et al., 2013; Fernandez et al., 2016; Gilpin et al., 2012; Serlin and Torregrossa, 2015; Toalston et al., 2015). In addition, adolescent alcohol exposure increases adult sensitivity to alcohol-induced learning deficits (Risher et al., 2013; White et al., 2000). Currently, the implications of disinhibited DLS function during adolescence are less clear. Interestingly, adolescent rats have been reported to be resistant to development of habitual response strategies (Serlin and Torregrossa, 2015), despite consuming more ethanol than adults during self-administration. However, in the same study, rats that initiated alcohol self-administration during adolescence showed higher intake of self-administered alcohol once they reached adulthood compared with rats that began self-administration during adulthood followed by the same abstinence period. Thus, it is possible that adaptations in mGlu₂-LTD in the DLS are more likely to influence behavioral responses to alcohol later in life than within the adolescent period.

In addition to age-specific effects, variables such as sex and strain of the experimental subjects are likely to impact vulnerability to alcohol-induced adaptations in presynaptic regulation of neurotransmission. With regard to sex, there are well known differences between female and male mice in alcohol intake and other alcohol-related behaviors (Erol and Karpayak, 2015; Guizzetti et al., 2016). The current study was limited to male subjects, and further studies will be necessary to determine the impact of adolescent alcohol exposure on mGlu₂ function in female mice. Furthermore, exploration of alcohol-induced adaptations

in GPCR function in rats or different mouse strains could provide additional insight into common or divergent effects of ethanol on mGlu₂ function, as different mouse strains used to study alcohol-related behaviors exhibit divergent responses to the rewarding, reinforcing, aversive, and stimulating effects of alcohol (Halladay et al., 2017). Differential adaptations in synaptic modulation in response to alcohol exposure could contribute to observed strain differences on a variety of alcohol-related behaviors.

Several preclinical studies in rats and mice have implicated the group II mGlu receptor mGlu₂ as a target for reducing alcohol seeking and consumption. For example, genetic analysis revealed that selectively-bred alcohol-preferring (P) rats are uniformly homozygous for a variant of *Grm2* harboring a premature stop codon (*Grm2**407), and this allele is prevalent in other lines of rats selectively bred for high alcohol consumption as well (Wood et al., 2016; Zhou et al., 2013). Pharmacological inhibition of group II mGlu receptors in rats increases alcohol self-administration, and genetic deletion of *Grm2* in mice increases alcohol consumption and preference in a two-bottle choice alcohol drinking paradigm (Zhou et al., 2013), suggesting that endogenous activation of mGlu₂ constrains alcohol-consuming behaviors under normal conditions. Pharmacological studies using agonists of mGlu₂ and –3 or positive allosteric modulators selective for mGlu₂ show that activation of these receptors decreases voluntary drinking (Griffin et al., 2014), reduces the discriminative stimulus properties of alcohol (Cannady et al., 2011), decreases alcohol self-administration, and reduces conditioned reinstatement of alcohol seeking following extinction (Augier et al., 2016; Backstrom and Hyytia, 2005; Kufahl et al., 2011; Windisch and Czachowski, 2018; Zhao et al., 2006). In addition, downregulation of *Grm2* has been reported in the infralimbic cortex in a rat model of alcohol dependence, and lentivirus-mediated restoration of mGlu₂ expression reduced conditioned reinstatement of alcohol seeking in alcohol-dependent rats (Meinhardt et al., 2013). Interestingly, the same study reported reduced *Grm2* levels in the anterior cingulate cortex in postmortem samples obtained from humans diagnosed with AUD when compared with age- and postmortem interval-matched controls, suggesting that an mGlu₂ hypofunction hypothesis has translational relevance (Meinhardt et al., 2013). Collectively, these findings support the idea that reduced mGlu₂ function could promote high levels of drinking, and that enhancing mGlu₂ activity could reduce alcohol intake and seeking in humans. However, little is known about mGlu₂ effects in specific circuits relevant to habitual alcohol seeking and consumption. Our report that alcohol impairs mGlu₂-LTD in the DLS extends these findings to implicate circuitry specifically relevant to habitual response strategies that could support uncontrolled alcohol seeking following adolescent alcohol exposure.

We used electrical stimulation of glutamatergic inputs to MSNs to broadly assess the impact of alcohol on mGlu₂ function on inputs to each subregion of the dorsal striatum. The specific glutamatergic inputs to the striatum that are impacted by adolescent CIE remain to be identified, and the subset of inputs affected could have important implications for the behavioral relevance of impaired mGlu₂-LTD. Few investigations have focused on the relevance of reduced mGlu₂ expression or function in specific cortical regions. Reduced expression in the infralimbic cortex has been associated with increased vulnerability to cue-induced reinstatement of alcohol seeking (Meinhardt et al., 2013). Conversely, efforts to identify a role for prelimbic cortex mGlu₂ in voluntary drinking demonstrated that 40%

knockdown of mGlu₂ from this region was insufficient to increase drinking behavior in rats (Ding et al., 2017). Further studies focused on cortical regions projecting to the DLS will be necessary to pinpoint the affected cortical or thalamic afferents and to determine the behavioral ramifications of disinhibition of affected striatal inputs.

Our efforts to measure downregulation of mGlu₂ expression at both the mRNA and protein levels did not produce evidence that reduced receptor expression can account for alcohol-induced impairment of mGlu₂-LTD. This finding is in contrast to the report by Meinhardt et al. (2013) that CIE combined with alcohol self-administration reduces mGlu₂ expression in the infralimbic cortex of alcohol-dependent rats. Our medial PFC samples contained, but were not limited to, infralimbic cortex. Therefore, differences in the subregions evaluated, as well as differences in species and alcohol exposure paradigms, could explain this discrepancy. Several alternative mechanisms for reduced receptor function could underlie alcohol-induced impairment of mGlu₂-LTD. For example, alcohol could disrupt coupling of mGlu₂ to heterotrimeric G proteins or downstream signaling molecules that regulate LTD. This explanation is particularly attractive in light of our finding that in the DLS, alcohol differentially impacts the early vs. long-term inhibition of glutamate transmission by mGlu₂, which are likely to involve different complements of presynaptic signaling events. Further, reduced downstream signaling provides a parsimonious explanation for the effects of alcohol on other presynaptic GPCRs in the striatum, including CB1 receptors and mu opioid receptors. Another intriguing possibility is that alcohol disrupts the subcellular localization of mGlu₂ such that efficacy is impacted without an accompanying reduction in expression. Nanoscale localization of CB1 receptors in relation to the active zone and downstream effectors has been proposed as a critical determinant of neuromodulatory efficacy at hippocampal synapses (Dudok et al., 2015). mGlu₂ efficacy within presynaptic terminals could be similarly impacted by subtle differences in subcellular localization following alcohol exposure.

From a translational perspective, our finding that acute treatment of slices with a selective PAM of mGlu₂ can rescue mGlu₂-LTD after alcohol exposure has important implications for the consideration of mGlu₂ as a therapeutic target for AUDs. The authors of the previous study reporting an alcohol-induced downregulation of mGlu₂ reasoned that reductions in mGlu₂ expression would preclude the use of drugs targeting mGlu₂ in AUDs due to insufficient availability of the target (Meinhardt et al., 2013). Contrary to this assertion, we demonstrated that an mGlu₂ PAM can fully restore alcohol-induced deficits in mGlu₂-LTD in the dorsal striatum. Although our PAM rescue did not occur in the context of reduced receptor expression, others have demonstrated that in the absence of receptor reserve due to reduced expression, PAMs of mGlu receptors can increase the maximal efficacy of receptor activation to rescue impaired synaptic modulation (Gogliotti et al., 2017). Thus, pharmacological targeting of mGlu₂ to alleviate alcohol-induced shifts towards maladaptive action strategies related to DLS function remains a viable therapeutic strategy. Future studies directly examining the role of impaired mGlu₂ function in alcohol-induced behavioral adaptations such as increased use of habitual action strategies, and the ability of mGlu₂ PAMs to reverse maladaptive behaviors, will provide insight into the behavioral relevance of disrupted mGlu₂-LTD in the dorsal striatum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Alcohol impairs mGlu₂-mediated long-term depression in the dorsolateral striatum.
- Adolescent mice are uniquely vulnerable to alcohol effects on mGlu₂-LTD.
- LTD is rescued by an mGlu₂ positive allosteric modulator.
- Alcohol has modest effects on mGlu₂-LTD magnitude in the dorsomedial striatum.

Fig. 1. CIE during adulthood does not impair mGlu₂-LTD.

(a) Time course of LY379268 (100 nM, 5 min) effects on electrically-evoked EPSC amplitudes recorded in the dorsolateral striatum in slices from air or CIE mice; $n = 6$ (air) or 8 (CIE) cells per group. (b) Sample traces from baseline (left), early depression (middle), and long-term depression (right) from representative experiments. Scale bars: 100 pA, 50 ms (top) or 200 pA, 50 ms (bottom). (c) Summary of LY379268 effects on the early component of depression. (d) Summary of LY379268 effects on LTD magnitude. Data are normalized to the average baseline EPSC amplitude and shown as mean $\pm$ SEM.

Fig. 2. mGlu₂-LTD is impaired by CIE during late adolescence and is rescued by an mGlu₂ PAM.

(a,c) Time course of LY379268 (100 nM, 5 min) effects on EPSC amplitudes recorded from the dorsolateral striatum in slices from air or CIE mice in the absence (a) or presence (c) of the mGlu₂-selective positive allosteric modulator LY487379 (10 μ M, present throughout recording); $n = 4-9$ cells per group. (b,d) Sample traces from baseline (left), early depression (middle), and long-term depression (right) from representative experiments in presence of vehicle (b) or LY487379 (d). Scale bars: 100 pA, 50 ms (b, top, and d, top and bottom) or 200 pA, 50 ms (b, bottom). (e) Summary of LY379268 effects on the early component of depression. (f) Summary of LY379268 effects on LTD magnitude. * $p < 0.05$, Tukey's *post hoc* comparison. Data are normalized to the average baseline EPSC amplitude and shown as mean $\pm$ SEM.

Fig. 3. Adolescent CIE-induced impairment of mGlu₂-LTD does not require prolonged withdrawal.

(a) Time course of LY379268 (100 nM, 5 min) effects on EPSC amplitudes recorded from the dorsolateral striatum in slices from air or CIE mice; $n = 6$ cells per group. (b) Sample traces from baseline (left), early depression (middle), and long-term depression (right) from representative experiments. Scale bars: 100 pA, 50 ms (top) or 50 pA, 50 ms (bottom). (c) Summary of LY379268 effects on the early component of depression. (d) Summary of LY379268 effects on LTD magnitude. $*p=0.0072$, unpaired t test. Data are normalized to the average baseline EPSC amplitude and shown as mean $\pm$ SEM.

Fig 4. Acute application of ethanol to slices does not impair mGlu₂-LTD.

(a) Time course of LY379268 (100 nM, 5 min) effects on EPSC amplitudes recorded from the dorsolateral striatum in the presence of ethanol (40 mM, slices pretreated for at least one hour prior to recording and maintained throughout recording); $n = 7$. Data are normalized to the average baseline EPSC amplitude and shown as mean $\pm$ SEM. (b) Sample traces from baseline (left), early depression (middle), and long-term depression (right) from a representative experiment. Scale bars: 200 pA, 50 ms.

Fig. 5. Adolescent CIE reduces the magnitude of mGlu2-LTD in the dorsomedial striatum.

(a) Time course of LY379268 (100 nM, 5 min) effects on EPSC amplitudes recorded from the dorsomedial striatum in slices from air or CIE mice $n = 6$ (air) or 8 (CIE) cells per group. (b) Sample traces from baseline (left), early depression (middle), and long-term depression (right) from representative experiments. Scale bars: 200 pA, 50 ms (top) or 100 pA, 50 ms (bottom). (c) Summary of LY379268 effects on the early component of depression. $p=0.058$, unpaired t test. (d) Summary of LY379268 effects on LTD magnitude. $*p=0.037$, unpaired t test. Data are normalized to the average baseline EPSC amplitude and shown as mean $\pm$ SEM.

Fig. 6. No evidence for downregulation of Grm2 expression following adolescent CIE.

Relative expression of Grm2 RNA in cortical or thalamic samples obtained from air or adolescent CIE mice 3 days after last alcohol exposure. Blood ethanol concentrations for this group were 239.7 ± 10.3 mg/dL ($n = 8$). (a) Anterior insular cortex: air 0.018 ± 0.003 , $n = 8$; CIE 0.013 ± 0.001 , $n = 8$; $p = 0.13$, unpaired t test. (b) primary motor cortex (M1): air 0.030 ± 0.004 , $n = 7$; CIE 0.024 ± 0.002 , $n = 8$; $p = 0.18$, unpaired t test. (c) medial prefrontal cortex (prelimbic, infralimbic, anterior cingulate): air 0.019 ± 0.003 , $n = 7$; CIE 0.020 ± 0.003 , $n = 7$; $p = 0.80$, unpaired t test. (d) thalamus: air 0.026 ± 0.004 , $n = 8$; CIE 0.028 ± 0.004 , $n = 8$; $p = 0.76$, unpaired t test. Data are presented as mean $\pm$ SEM.

Individual data points are averaged from 3 replicates per sample.

Fig. 7. Adolescent CIE does not reduce mGlu₂ protein levels in the DLS or DMS.

(a) qPCR analysis of cortical tissue confirms that Grm2 expression is reduced in heterozygous mice and absent in mice homozygous for the knockout allele ($n = 4$ mice/group;). n.d. = not detected. Data points were averaged from 3 replicates per sample. (b) Immunoreactivity detected with an mGlu₂ antibody in dorsal striatum samples from wild type (WT), heterozygous (HET), or mGlu₂ knockout mice (KO). Data are normalized to immunoreactivity detected in the sample from the WT mouse. Approximately 17% of the total signal was still detectable in protein extract from the mGlu₂ knockout mouse. (c) Average mGlu₂ immunoreactivity (determined by integrating the chemiluminescence signal between 78kDa to 345 kDa and normalizing to total protein) detected in protein extracts from the DLS of air or adolescent CIE mice 3 days after last alcohol exposure (air $2.39 \times 10^7 \pm 0.25 \times 10^7$, $n = 9$; CIE $1.96 \times 10^7 \pm 0.08 \times 10^7$, $n = 8$; $p = 0.15$). (d) Representative electrophoretograms of DLS samples from air or CIE mice showing peaks at ~118 kDa and ~180 kDa. (e) Average mGlu₂ immunoreactivity detected in protein extracts from the DMS of air or adolescent CIE mice 3 days after last alcohol exposure (air $1.93 \times 10^7 \pm 0.09 \times 10^7$, $n = 9$; CIE $1.84 \times 10^7 \pm 0.08 \times 10^7$, $n = 8$; $p = 0.45$). (f) Representative electrophoretograms of DMS samples from air or CIE mice. Data in (a), (c), and (e) are presented as mean $\pm$ SEM. For (c) and (e), data points are averaged from two replicates per sample. Blood ethanol concentrations for this group were 211.4 ± 6.6 mg/dL ($n = 9$).